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ISOCARBOSTYRIL ALKALOIDS FROM *HAEMANTHUS KALBREYERI**

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Key Word Index—*Haemanthus kalbreyeri*; Amaryllidaceae; isocarbostyril alkaloids; 7-deoxypancratistatine; pancratistatine-2-*O*-β-D-glucoside; plant growth regulatory effects.

Abstract—Two new isocarbostyril alkaloids, 7-deoxypancratistatine and pancratiside (=pancratistatine-2-*O*-β-D-glucoside), were isolated from the resting bulbs of *Haemanthus kalbreyeri*. The structures of the two alkaloids were established by comprehensive spectral analyses and chemical transformations. The plant growth regulatory profile of these two alkaloids and three other isocarbostyril congeners, narciclasine, 7-deoxynarciclasine, and pancratistatine, is appraised. *Haemanthus kalbreyeri* provides a new source of the anti-tumour alkaloid, pancratistatine.

INTRODUCTION

The occurrence of narciclasine (**2**), pancratistatine (**4**) and related isocarbostyril alkaloids, in members of the family Amaryllidaceae, is of particular interest in view of their possible use as anti-tumour agents [1]. However, from what is known at the present time [2], it is difficult to postulate if these alkaloids are restricted only to a few Amaryllidaceae species endemic to certain parts of the World. Search for newer sources of these alkaloids is deemed to be warranted to ensure their availability and from the point of view of their significance in plant biochemistry. In the course of our ongoing studies about the catabolism and function of Amaryllidaceae alkaloids [3], we observed certain ontogenic variations in the occurrence and accumulation of narciclasine and 7-deoxynarciclasine, and some partially characterized isocarbostyril alkaloids in a number of *Haemanthus*, *Pancratium* and *Zephyranthes* species [4]. The isocarbostyril alkaloids were found, in appreciable amounts, in the roots of resting bulbs of *H. kalbreyeri* Bak., *P. biflorum* Roxb., *Z. flava* Roem. & Schult and *Z. rosea* Lindl. In the flowering bulbs these alkaloids were present only in traces. Their presence was detected by the characteristic UV and Tandem MS/ms spectra, HPLC retention times, and specific colour reactions, e.g. with potassium iodide–platinic chloride and Hansen reagents (for methylenedioxy group). Quantitation of the individual entities was possible by analytical HPLC. The present paper describes the natural occurrence of five isocarbostyril alkaloids in *H. kalbreyeri* in quantities sufficient for their complete characterization and their plant growth-regulatory profile.

RESULTS AND DISCUSSION

Extensive solvent-gradient separation followed by column chromatography, preparative TLC and HPLC of the aqueous methanol extractives of roots of resting bulbs of *H. kalbreyeri* afforded 10 alkaloids, viz. haemanthamine [5], haemanthidine [5], hippadine [5], kalbretorine [5], lycorine [5], narciclasine [5], 7-deoxynarciclasine [2], pancratistatine [2], 7-deoxypancratistine and pancratiside. Among these, the two last named compounds are new alkaloids. Characterization of the new alkaloids only is described here.

7-Deoxypancratistatine

This compound, $C_{14}H_{15}NO_7$ (by elemental analyses and M^+) was obtained as a microcrystalline solid, mp 310–314° (dec), which was optically active. It exhibited UV and IR spectra similar to those of an isocarbostyril alkaloid [5]. However, that this alkaloid was different from pancratistatine was evident from its molecular formula, longer retention time in analytical HPLC and ^1H NMR spectrum which exhibited two Ar-H singlet signals instead of the one shown by pancratistatine (see Experimental). Acetylation of the compound with acetic anhydride–pyridine at 60°, for 2 hr, afforded a mixture of tetra- and tri-*O*-acetyl derivatives. The latter was found to be identical with the triacetate derivative of 7-deoxynarciclasine. This was conceivably produced by the loss of a molecule of acetic acid (Scheme 1). The parent alkaloid was converted into aroyllycoricidine [6] on treatment with methanol–hydrochloric acid. On the basis of the above observations, the 7-deoxypancratistatine structure (**3**) was assigned to this alkaloid.

It is interesting to note that like the narciclasine-7-deoxynarciclasine pair [6], pancratistatine also co-occurs with its 7-deoxy analogue in *H. kalbreyeri* and in *P. biflorum*, *Z. flava* and *Z. rosea* [4]. This observation is significant from the point of view of catabolism of vittatine and equivalents into the isocarbostyril alkaloids [7].

Pancratiside

This compound, $C_{20}H_{25}NO_{13}$, obtained as a light-brown hygroscopic solid, was optically active. It responded to ferric and benzidine metaperiodate tests for phenolic glycosides. In the EIMS, it fragmented before

*Part 29 in the series "Chemical Constituents of Amaryllidaceae". For Part 28 see ref [11].

exhibiting any molecular ion peak. However, identifiable fragment ion peaks appeared due to the aglucone (*m/z* 325) and the glucone (*m/z* 163, [M−17]) moieties. On enzymatic hydrolysis with emulsin, it gave pancratistatine (**4**) and D-glucose. Pancratiside did not give a consistent combustion analysis due to irregular solvation of the molecule. Attempts to prepare a crystalline derivative were also unsuccessful because of its facile conversion into fully aromatic compounds similar to those of kalbreclasine [5]. Pancratiside, however, formed an amorphous heptaacetate on treatment with acetic anhydride–triethylamine at room temperature. The heptaacetate exhibited an identifiable $[M+H]^+$ peak in its CIMS. The ^1H NMR spectrum of the heptaacetate, in $CDCl_3$, suggested that all the acetyl groups (21 H) were attached to the alcoholic hydroxyl functions. The presence of a chelated phenolic OH group (exchangeable with D_2O) in this compound was also discerned. Additionally, the ^1H NMR spectrum showed one aromatic (1H), one methylenedioxy (2H) and thirteen aliphatic (13 H) protons associated with the aglucone and the glucone moieties. The heptaacetate when heated (95°) with acetic anhydride, afforded kalbreclasine hexaacetate (**6**, OAc instead of alcoholic OH groups) [5] as a minor product. This was produced by the loss of one molecule of acetic acid from the heptaacetate (Scheme 1). On the basis of the above observations, pancratiside was assigned the pancratistatine-2-*O*-*β*-D-glucoside structure (**5**).

The free alkaloids, narciclasine (**2**), pancratistatine (**4**), and 7-deoxypancratistatine, in low concentrations ($1-5 \times 10^{-4}$ M), were found to inhibit germination of seeds and growth of roots in both producer and non-producer plant species. The *O*-glucosides (**5** and **6**) of pancratistatine and narciclasine, on the other hand, markedly promoted seed germination and root growth, in similar concentrations, in both producer and non-producer species. Similar observations were recorded before in respect of lycorine-lycorine-1-*O*-*β*-D-glucoside pair [8, 9]. Such distinctly different biological effects recorded for the free and glyco-alkaloids are an advance in understanding their role in the growth regulatory mechanisms of Amaryllidaceae species.

EXPERIMENTAL

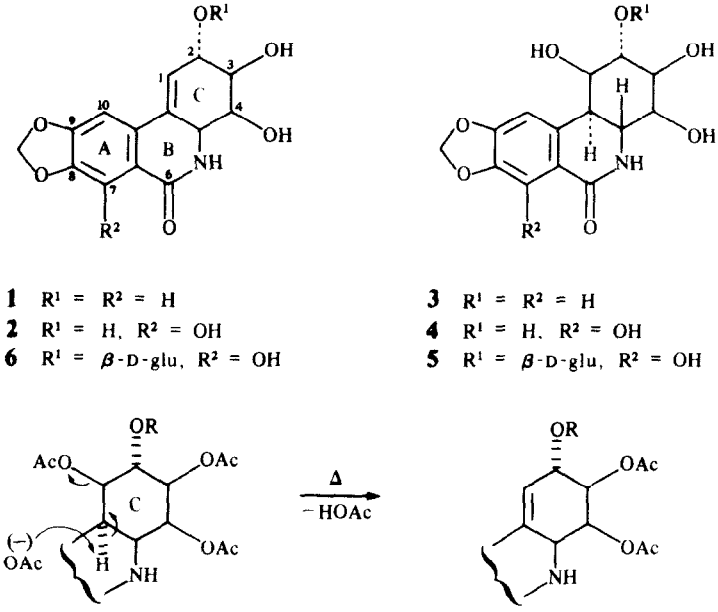
General procedures were the same as reported recently [10].

Plant. Haemanthus kalbreyeri Bak., cultivated in the Banaras Hindu University Campus, Varanasi, was identified by Professor S. K. Roy, Department of Botany, Banaras Hindu University. The plant materials were collected in two consecutive years during Dec.–Jan. and processed separately.

Isolation procedure. Fresh roots (5.2 kg) were macerated in MeOH (6 l), EDTA (0.1 ml) was added to arrest the enzymic activity, and the macerate was kept for 3 days at room temp. It was filtered and the filtrate concd under red. pres. to give a viscous brown residue, which was extrcd in succession with petrol. (fraction A), EtOAc (fraction B), and *n*-BuOH (fraction C). Treatment of fractions A and B, as before [5], afforded four known alkaloids, haemanthamine (55 mg), haemanthidine (28 mg), kalbretorine (17 mg), and lycorine (41 mg).

Treatment of fraction C. This fraction was evapd to give a brown residue (26 g). A portion (2.5 g) was dissolved in MeOH–dioxane (1:1, 50 ml) and filtered. The filtrate was kept at 5° overnight when a light-brown gummy material separated. The supernatant (fraction C_1) was decanted and the gummy material (fraction C_2) was washed with EtOAc. The EtOAc washings were combined with fraction C_1.

Treatment of fraction C_1. The residue from this fraction was dissolved in MeOH, the MeOH soln was combined with Si gel (BDH), carefully dried under vacuum, and charged on a Si gel chromatographic column (30 × 3 cm). Elution was carried out with $CHCl_3$, $CHCl_3$–MeOH (99:1, 95:5, 90:10), and MeOH. Fractions (100 ml) were collected and monitored by analytical TLC. The early $CHCl_3$–MeOH (95:5) eluates afforded a mixture of narciclasine (**2**, 23 mg) and 7-deoxynarciclasine (**1**, 9 mg) which were separated by prep. TLC as before [5].



Scheme 1. Formation of aromatic-conjugated isocarbostyrils during acetylation.

Pancratistatine (**4**). The later $CHCl_3$–MeOH (9:1) and the MeOH eluates were combined and evapd to give a mixture of pancratistatine and 7-deoxypancratistatine as the two major alkaloids. Crystallization of the mixture from MeOH–AcOH afforded pancratistatine as straw-coloured micro-crystals (82 mg), mp 307–310° (dec.); $[\alpha]_D^{28}$ +42.8° (DMSO; *c* 0.55); UV: λ_{max} (MeOH) nm (log ε) 219 sh, 233 (4.31), 278 (3.89) 308 sh; R_t(retention time in min) 4.2 [Waters Associates; μ Bondapak C_{18} column; developer, 0.01 M$(NH_4)_2HPO_4$–MeCN (7:3); flow rate 2 ml/min; detector, UV 440/254 nm; 0.1 a.u.f.s.]; R_t3.4 (developer, MeOH–H_2O; 8:2); MS: *m/z* (rel. int. %) 325 (M^+, 22), 307 (25), 289 (11),247 (100) [M^+(by accurate mass measurement), 325.079. $C_{14}H_{15}NO_8$ requires M^+, 325.0793]. The physical and spectral properties of the alkaloid were consistent with those reported for pancratistatine in the lit. [2].

7-Deoxypancratistatine (**3**). The MeOH–AcOH mother liquor, after separation of pancratistatine, was passed through a short column of florisil. Elution with $CHCl_3$–MeOH (1:1) afforded 7-deoxypancratistatine as a straw coloured amorphous solid (14 mg), mp 310–314° (dec.); $[\alpha]_D^{28}$ + 53.7° (DMSO; *c* 0.42); R_t 4.6 (MeOH–H_2O, 8:2); λ_{max} (MeOH) 222 (4.30), 262 sh (3.67), 270 (3.88), 305 (3.51); γ_{max}(KBr) cm^{-1} 3500–3400 (*br*), 1675, 1660, 1628, 1610, 1038, 1025, 940, 928; MS: *m/z* 309 (M^+, 48), 291 (23), 281 (11), 280 (5), 273 (9), 267 (7), 255 (14), 231 (72), 230 (100); ^{1}H NMR (DMSO-d_6); δ 6.98 (1H, *s*, H-10), 6.61 (1H, *s*, H-7), 6.20 (2H, *s*, OCH_2O), 3.5–4.4 (complex 6H), 4.7–5.5 (5H, exchangeable with D_2O) [M^+ (by accurate mass measurement), 309.0842. $C_{14}H_{15}NO_7$ requires M^+, 309.0844]. Acetylation of the alkaloid with Ac_2O–pyridine at 60°, for 2 hr, afforded a mixture (*ca* 1:1) of tetra- and tri-*O*-acetyl compounds. These were separated by prep. TLC (Si gel G; $CHCl_3$–MeOH, 19:1). The less polar compound was found to be identical with tri-*O*-acetyl-7-deoxynarciclasine, mp and mmp 201–203°; ^{1}H NMR ($CDCl_3$): δ 7.52 (1H, *s*, H-7), 6.98 (1H, *s*, H-10), 6.11 (2H, *s*, OCH_2O), 2.02–2.05 (9H, OAc); MS: *m/z* 417 (M^+, 28), 358 (9), 357 (14), 231 (100), 230 (88) [M^+(by accurate mass measurement), 417.1058. $C_{20}H_{19}NO_9$ requires M^+, 417.1054]. These properties were consistent with those of tri-*O*-acetyl-7-deoxynarciclasine (= tri-*O*-acetyllycoricidine) reported in the lit. [6]. The tetra-*O*-acetyl-7-deoxypancratistatine as obtained from the more polar R_F zone ~0.4, as a micro-crystalline solid, mp 228–233° (dec); *m/z* 477.1266. $C_{22}H_{23}NO_{11}$ requires 477.1264.

Treatment of fraction C_2. The brown gummy material was dissolved in H_2O and EtOH was added (total concn 50%) when pancratiside was pptd as an amorphous solid. The process was repeated several times to obtain practically pure pancratiside as a light brown hygroscopic solid (102 mg). This was dissolved in H_2O and passed through a column of Sephadex LH-20. The H_2O–MeOH (1:1) eluates, on evapn, afforded the pure glycoalkaloid, mp 203–218° (foaming, dec.); $[\alpha]_D^{28}$ + 22.5° (H_2O; *c* 1.2); R_t 10.8 (MeOH–H_2O, 8:2); heptaacetate with Ac_2O–Et_3N, at room temp., crystallized from hexane–EtOH as colourless micro-crystals, mp 122–126°; IR: ν_{max} (Nujol) cm^{-1} 1748, 1735, 1718, 1662, 1612, 1218, 935; CI MS: *m/z* 782 $(M+H)^+$; EI MS: *m/z* 451 (M^+ – tetraacetylglucosyl, 18), 450 (11), 392 (7), 391 (12), 331 (100); ^{1}H NMR ($CDCl_3$): δ 13.10 (1H, strongly chelated OH, exchangeable with D_2O), 6.55 (1H, *s*, H-10), 6.11 (2H, *s*, OCH_2O), 4.2–5.6 (13 H), 1.99–2.05 (21H, OAc). (Found: C, 51.78; H, 5.23; N, 1.57. $C_{34}H_{39}NO_{20}$ requires C, 52.2; H, 5.0; N, 1.8).

The heptaacetate on heating with Ac_2O, at 90°, under N_2 atmosphere, gave kalbreclasine hexaacetate (by prep. TLC), mp and mmp 198–200° (co-TLC, IR, ^{1}H NMR [5].

Growth-regulatory effects. The effects of pancratistatine (**4**) and pancratiside (**5**) on the germination of seeds of *Zephyranthes flava* and radish were examined. Seeds were sown on the test compound-treated filter paper in Petri dishes, 20 Petri dish and 2 dishes per treatment; H_2O was added, and the dishes were wrapped in foil. The seeds were incubated at 27°. For every two treatments, a blank control was added. The rate of germination of the seeds was observed every 12 hr up to 96 hr. Forty to 50% of the control seeds were germinated after 36 hr. During this period, while 100% of the pancratiside-treated (1×10^{-4} M) seeds were germinated, none of the pancratistatine-treated (1×10^{-4} M) seeds germinated. Furthermore, pancratistatine continued to inhibit the germination of both *Z. flava* and radish seeds up to 60 hr; thereafter only 5–10% of seeds were germinated. The effects of the two alkaloids (**4** and **5**) were also tested on the development of roots and aerial parts of *Allium cepa*, *Pancratium biflorum* and *Z. flava*. In a typical experiment, 100 bulbs of *Z. flava* were surface-sterilized (0.1%NaOCl) and the bulbs were divided into 10 groups of *ca.* equal wt. Five groups were soaked (1 hr) in Pi buffered saline (PBS, 0.15 N, NaCl, pH 7.2) soln of the test compound (1×10^{-5} M) and placed in a moist chamber at 27° under aseptic conditions. An equal number of bulbs, treated with only the vehicle (PBS) were similarly kept as control. Growth and elongation of the roots were recorded up to 144 hr at 24 hr intervals. The rate of growth of roots was found to be maximum between 72 and 96 hr. At the end of this period, pancratiside was found to considerably promote the root growth (235% over the control; $n = 10$; $P < 0.001$; χ^2 (Chi square) significance) and elongation (177% over the control; $P < 0.01$). The number of root hairs was also significantly higher (164%) in this group. Pancratistatine, on the other hand, significantly inhibited both root growth and stem elongation (70–95% less in relation to the control groups). Both these alkaloids, however, completely prevented the emergence of leaves in *P. biflorum* and *Z. flava* (bulbs) up to 144 hr and, thereafter, continued to prevent the growth of leaves in these species for several weeks.

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